Transcriptional response of *Desulfatibacillum alkenivorans* AK-01 to growth on alkanes: insights from RT-qPCR and microarray analyses

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Abstract

Microbial transformation of n-alkanes in anaerobic ecosystems plays a pivotal role in biogeochemical carbon cycling and bioremediation, but the requisite genetic machinery is not well elucidated. *Desulfatibacillum alkenivorans* AK-01 utilizes *n*-alkanes (C_{13} to C_{18}) and contains two genomic loci encoding alkylsuccinate synthase (ASS) gene clusters. ASS catalyzes alkane addition to fumarate to form methylalkylsuccinic acids. We hypothesized that the genes in the two clusters would be differentially expressed depending on the alkane substrate utilized for growth. RT-qPCR was used to investigate *ass*-gene expression across AK-01's known substrate range, and microarray-based transcriptomic analysis served to investigate whole-cell responses to growth on n-hexadecane versus hexadecanoate. RT-qPCR revealed induction of ass gene cluster 1 during growth on all tested alkane substrates, and the transcriptional start sites in cluster 1 were determined via 5′RACE. Induction of ass gene cluster 2 was not observed under the tested conditions. Transcriptomic analysis indicated that the upregulation of genes potentially involved in methylalkylsuccinate metabolism, including methylmalonyl-CoA mutase and a putative carboxyl transferase. These findings provide new directions for studying the transcriptional regulation of genes involved in alkane addition to fumarate, fumarate recycling and the processing of methylalkylsuccinates with regard to isolates, enrichment cultures and ecological datasets.

Keywords: anaerobic; alkane; transcription; microarray; alkylsuccinate synthase

INTRODUCTION

Anaerobic alkane oxidation contributes to the natural attenuation of oil contaminants (for reviews, see Joye, Teske and Kostka 2014; Kimes *et*

al. 2014; King *et al*. 2015), oil souring by alkane-utilizing, sulfate-reducing organisms in oil reservoirs (Gieg, Jack and Foght 2011), methane removal from oceanic systems (Hinrichs and Boetius 2002; Reeburgh 2007) and biogenic methane production in coal beds via syntrophic, hydrocarbon-degrading consortia (Wawrik *et al*. 2012). Despite the importance of these processes, there are only 19 anaerobic, alkane-oxidizing isolates that have been characterized to date (Webner 2012; Schouw *et al*. 2016), with few having complete genomes that are publicly available. *Desulfatibacillum alkenivorans* AK-01 is a sulfate-reducing bacterium that catalyzes the anaerobic oxidation of alkanes, alkenes, alcohols, organic acids and fatty acids (So and Young 1999a). The activation of alkanes in AK-01 occurs via alkane addition to fumarate (i.e. 'fumarate addition'), which was first proposed by So and Young (1999b) and later substantiated via metabolite analysis (Callaghan *et al*. 2006) (Fig. 1a). This led to the hypothesis that a glycyl radical enzyme (GRE) similar to benzylsuccinate synthase (BSS), which catalyzes the addition of aromatic hydrocarbons to fumarate (Heider and Schühle 2013), mediates fumarate addition in AK-01 and other alkane-utilizing isolates (Rabus *et al*. 2001; Callaghan *et al*. 2006). This hypothesis was supported by the detection of two genes in AK-01 that encode the catalytic (i.e. alpha) subunits of putative glycyl radical-type enzymes similar to BSS (Callaghan *et al*. 2008). Subsequent analysis of the AK-01 genome revealed two corresponding loci that contain additional gene features hypothesized to be the requisite activases and necessary subunits of putative GREs, which were named alkylsuccinate synthases (ASS) 1 and 2 (Callaghan *et al*. 2008, 2012) (Fig. 1b). A similar enzyme, (1-methylalkyl)succinate synthase (MAS), was identified in the alkane-utilizing, denitrifier '*Aromatoleum*' sp. HxN1 (Grundmann *et al*. 2008), and a subsequent study of HxN1 revealed that it contains two identical *mas* operons (Webner 2012). With regard to AK-01, a preliminary proteomic investigation demonstrated AssA1 expression when *D. alkenivorans* AK-01 was grown on *n*-hexadecane versus hexadecanoic acid (Callaghan *et al*. 2008). Specifically, AssA1 could be uniquely matched to predicted tryptic fragments, whereas AssA2 could not. However, because this study only addressed the expression of the alpha subunits under one alkane-amended condition and did not investigate the expression of the other putative subunits during growth on AK-01's complete *n*-alkane substrate range, further investigation is still warranted.

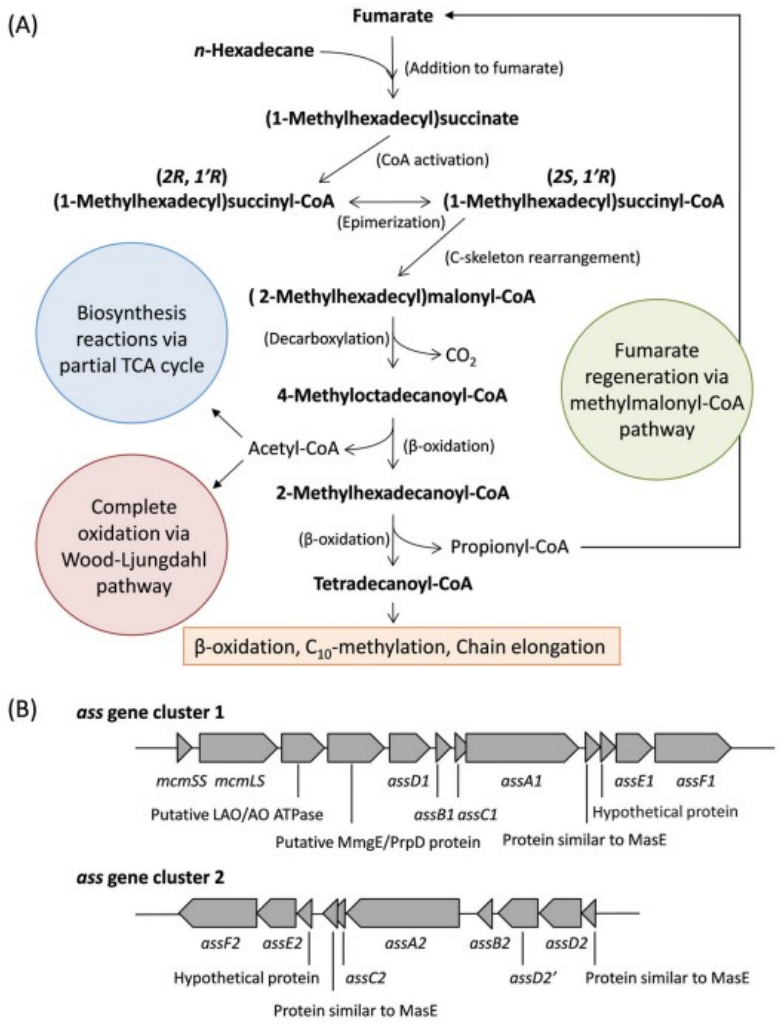

Figure 1. (A) Proposed pathway of *n*-hexadecane metabolism in *D. alkenivorans* AK-01 based on previous metabolite profiling experiments (Callaghan *et al.* 2006) and genome analysis (Callaghan *et al.* 2008). Genome analysis revealed two loci consisting of *ass* genes. (B) The corresponding JGI locus tag numbers for the genes in *ass* gene clusters 1 and 2 (left to right) are Dalk_1724 through Dalk_1735 and Dalk_2194 through Dalk_2203, respectively.

The discovery of ASS/MAS in AK-01 and HxN1 has facilitated the interrogation of other isolates, enrichment cultures and hydrocarbon-impacted ecosystems for the presence of genes potentially involved in alkane activation. For example, characterization studies and genome surveys of alkane-utilizing bacteria that carry out fumarate addition have shown that *D. aliphaticivorans* CV2803^{T} (Cravo-Laureau *et al*. 2004; Cravo-Laureau *et al*. 2005) has two alkylsuccinate synthases (https://img.jgi.doe.gov/cgi-bin/er/main.cgi); the sulfate-reducers *Desulfosarcina/Desulfococcus* strain BuS5 (Kniemeyer *et al*. 2007) (www.ncbi.nlm.nih.gov.), *Desulfatibacillum* sp. Pnd3 (Aeckersberg, Rainey and Widdel 1998) and *Desulfothermus naphthae* TD3 (Rueter *et al*. 1994) appear to have one copy of MAS (Webner 2012); and the nitrate-reducer '*Aromatoleum*' sp. OcN1 (Ehrenreich *et al*. 2000) also has only one copy of MAS (Webner 2012). Interestingly, there is an expanding list of *ass* genes that have been detected in the assembled genomes of '*Smithella*'-like

organisms identified in methanogenic, alkane-utilizing consortia (Embree *et al*. 2014; Tan, Nesbø and Foght 2014; Tan *et al*. 2014; Marks *et al*. 2015). Although not isolated, transcriptomic data suggest that these organisms are the key players mediating alkane transformation via fumarate addition (Embree *et al*. 2014; Tan, Nesbø and Foght 2014; Marks *et al*. 2015). Ultimately, these studies aim to provide context for elucidating the complex microbial ecology of hydrocarbon-impacted systems. To that end, *bssA* and *assA* have been used as biomarkers indicative of anaerobic hydrocarbon degradation via fumarate addition mechanisms (Lueders and von Netzer 2014). Specifically, *in situ* investigations have detected *assA* in estuarine systems, deep-sea sediments, groundwater and coal beds (Callaghan *et al*. 2010; Wawrik *et al*. 2012; Kimes *et al*. 2013; von Netzer *et al*. 2013; Johnson *et al*. 2015), providing strong evidence for the potential utilization of alkane substrates in the environment. These efforts have also been employed in conjunction with metabolomic techniques (for reviews, see Agrawal and Gieg 2013; Callaghan 2013).

The number of microbial ecology investigations aimed at providing insight into hydrocarbon-impacted ecosystems (Kostka *et al*. 2014 and references therein) continues to expand due to the biogeochemical and economic importance of anaerobic hydrocarbon biotransformation processes, coupled with affordable next-generation sequencing technologies. The interpretation of ecological data is facilitated significantly by the knowledge base generated from the physiological and biochemical characterization of environmentally relevant isolates such as AK-01. This is especially true for molecular studies, which require quality annotation of large, metagenomic and metatranscriptomic datasets. With respect to anaerobic alkane oxidation, the elucidation of how the diversity of ASS proteins relates to the phylogeny of microorganisms and their respective hydrocarbon substrate ranges is still needed to provide context for interpreting the presence of such genes in environmental samples. Historically, this effort has been impeded both by the paucity of genetic systems for requisite anaerobic, alkane-utilizing isolates and the very slow-growing lifestyles of these bacteria. Given these challenges, the study herein focused on the transcription of the two *ass* gene clusters in AK-01 as well as genome-wide transcriptomic patterns in response to alkane metabolism. Based on the preliminary proteomic study cited above (Callaghan *et al*. 2008), we hypothesized that the genes in the two clusters would be differentially expressed depending on the alkane substrate and that a distinct global pattern of gene expression in response to alkane metabolism would be observed, providing insight into the genetic machinery that may be involved in the further degradation of methylalkylsuccinic acids.

MATERIALS AND METHODS

Cultivation

Cultures of *D. alkenivorans* AK-01 were grown in sulfate-reducing media as previously described (Callaghan *et al*. 2006) amended with either 1 mM of *n*-alkane (C_{13}–C_{18}) or 1 mM of *n*-fatty acid substrate (13:0–18:0) and 8.5 mM sulfate. Sulfate loss was monitored via ion chromatography using an ion chromatograph (ICS-1100, Dionex Corp., Sunnyvale, CA) equipped with an ion-exchange column system (IonPac® AS23 4 × 250 mm analytical column and an IonPac AG23 4 × 50 mm guard column) (Dionex Corp., Sunnyvale, CA) and a conductivity detector. The eluent contained 4.5 mM Na_2CO_3 and 0.8 mM $NaHCO_3$, and the flow rate was 1 ml min^{-1}.

RNA extraction, DNase digestion and cDNA synthesis

Cultures were sampled during exponential growth when 70%–80% sulfate loss was observed (i.e. cultures were actively growing and sufficient biomass had accumulated). Aliquots of triplicate biological cultures were filtered onto 0.45 μm Supor filters (Supor®, PALL Life Sciences). Filters were stored in RLT buffer (RNeasy® mini kit, Qiagen, Valencia, CA) containing 1% v/v of β-mercaptoethanol, immediately frozen in liquid N_2, and stored at −80°C until RNA extraction. For extraction, samples were thawed and ca. 100 mg of 0.1 mm sterile, muffled glass disruption beads (Research Products International Corp, Mount Prospect, IL) were added to each tube. Cells were homogenized using a mini bead-beater (Biospec Products, Inc., Bartlesville, OK) for 1 min, incubated on ice for 5 min and homogenized a second time for 1 min. Extraction of total RNA was performed using the Qiagen RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Contaminating DNA was removed with RQ1 RNase-free DNase (Promega, Madison, WI). The absence of DNA contamination was confirmed via PCR amplification using bacterial 16S PCR primers 27F (5′ AGAGTTTGATCMTGGCTCAG3′) and 519R (5′GWATTACCGCGGCKGCTG3′) (Lane 1991). The cDNA was synthesized using a SuperScript®VILO cDNA synthesis kit (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Total RNA and cDNA were quantified using a Qubit® 2.0 Fluorometer (Life Technologies, Grand Island, NY) via the Qubit RNA BR and ssDNA assay kits, respectively (Life Technologies, Grand Island, NY).

Cotranscription experiments and RT-qPCR

As mentioned above, a prior proteomic study demonstrated AssA1 expression, but not AssA2 expression, when *D. alkenivorans* AK-01 was grown on *n*-hexadecane versus hexadecanoic acid (Callaghan *et al*. 2008). Therefore, the intergenic regions of genes adjacent to *assA1* were investigated via RT-PCR to obtain information regarding the potential cotranscription of genes that may form a putative operon (RT-PCR primers listed in Table S1, Supporting Information). RNA was extracted from triplicate *n*-hexadecane and *n*-heptadecane-grown cultures, reverse transcribed and PCR amplified as described above. PCR product sizes were evaluated via gel electrophoresis and sequenced to confirm the presence of

transcript across each respective intergenic region. This effort identified 11 genes that are potentially cotranscribed with *assA1* (see Results), and these were targeted for RT-qPCR experiments. RT-qPCR experiments for *ass* gene cluster 2 were focused on the respective gene copies that were shown to be cotranscribed in cluster 1. PCR primers were designed for the selected genes in both loci (Tables S2 and S3, Supporting Information). RT-qPCR primers were tested for cross-reactivity via PCR of AK-01 genomic DNA (gDNA) and sequencing of the PCR products. Optimum annealing temperatures were determined empirically via gradient PCR, by choosing the highest annealing temperature for which no loss of product was observed in gel electrophoresis. Reactions were performed in 30-µl volumes containing 10 ng of cDNA, 15 µL of Power SYBR green qPCR mix (Life Technologies, Grand Island, NY) and 330 nM of each primer. Reactions were carried out in a 7300 Real Time PCR Machine (Life Technologies, Carlsbad, CA) with the following conditions: initial denaturation at 95°C for 60 s was followed by forty cycles of 95°C for 30 s, 60 s at the respective annealing temperature and 72°C for 60 s. Standard curves were generated using *D. alkenivorans* AK-01 DNA, and RT-qPCR data were normalized to *gyrB* expression.

Transcriptional start sites

Based on the RT-qPCR experiments (see Results), putative transcriptional start sites in *ass* gene cluster 1 were investigated. Total RNA was extracted from two biological replicates of *n*-hexadecane-grown cultures. RNA extraction, quantification, DNase digestion and confirmation of the absence of DNA contamination were performed as described above. 5′-Rapid amplification of cDNA ends (5′-RACE) was conducted using the 5′/3′ RACE kit (2nd Generation) (Roche, Mannheim, Germany) following the manufacturer's instructions. PCR products were cloned into the pCRII vector using the Dual promoter TA® cloning kit (Life Technologies, Carlsbad, CA) and transformed into One Shot® Top10F’ chemically competent *Escherichia coli* as recommended by the manufacturer. Colonies were picked into 96-well microtiter plates, and insert sizes were screened via colony PCR using M13 primers and then sequenced.

Microarray design

Custom DNA microarrays were designed by Roche NimbleGen based on the complete genome sequence of *D. alkenivorans* AK-01 (GenBank Accession number: NC_011768). All annotated open reading frames (ORFs) were extracted and used to design up to eight 60-mer oligonucleotide probes for each ORF. A total of 5188 ORFs were targeted with unique sets of probes. Sixty-four ORFs could only be represented by ambiguous duplicates and were disregarded in the downstream analysis. All probes were laid down in triplicate, yielding a maximum of 24 unique probes per ORF on each array.

cDNA synthesis, amplification and labeling

RNA originating from the same triplicate *n*-hexadecane and hexadecanoic-acid-grown cultures used for RT-qPCR experiments was amplified and Cy5-labeled (GE Healthcare, Piscataway, NJ) by random priming via a modified protocol of Gao *et al*. (2007). RNA extracts (200–300 ng) were dried using a vacuum centrifuge and resuspended in 10 µl of RNase free water. One microliter of T7-N6 primer (AATTGTAATACGACTCACTATAGGGNNNNNN; 100 µM stock) (Gao *et al*. 2007) was added, samples were denatured at 65°C for 10 min and immediately transferred to ice. cDNA was synthesized using a SuperScript Double Stranded cDNA Synthesis kit (Life Technologies, Carlsbad, CA) with 200 units of SuperScript II reverse transcriptase, 1 µl of linear acrylamide (0.1 µg μl^{-1}) (Ambion, Austin, TX) and of 1 µl of RNAse inhibitor (Life Technologies, Carlsbad, CA). Second strand synthesis and end repair were performed in 150-µl reaction volumes at 16°C as recommended by the manufacturer. Samples were extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and subsequently with chloroform:isoamyl alcohol (24:1, v/v) and precipitated.

The remaining double-stranded cDNA sample was transcribed via *in vitro* transcription using a MEGAscript® *In Vitro* Transcription kit (Ambion, Austin, TX) according to the manufacturer's recommendations. RNA was purified using an RNAeasy purification kit (Qiagen, Valencia, CA) and quantified. RNA labeling was performed in 16.5 µl reactions containing 5.1 µg of amplified antisense RNA (aRNA), 3.3 µl of random primers (750 µg ml^{-1} random octamers) (Invitrogen BioPrime DNA Labeling System) (Life Technologies, Carlsbad, CA) and RNAse free water. After denaturation at 65°C for 10 min, samples were placed on ice. RT labeling mix was prepared from reagents in a SuperScript Double Stranded cDNA Synthesis kit (Life Technologies, Carlsbad, CA) and contained 6 µl of 5X RT-buffer, 3 µl of 0.1 M DTT, 1.5 µl of dNTP mix, 1 µl of RNAse inhibitor and 1 µl of 1mM Cy5-dUTP in 1mM phosphate buffer (GE Healthcare, Piscataway, NJ). The dNTP mix was prepared from 69.5 µl of RNAse free water, 10 µl of dATP, 10 µl of dGTP, 10 µl of dCTP and 0.5 µl of dTTP (100 mM stocks, Invitrogen). After 2 min of equilibration in the dark, 500 units of SuperScript II Reverse Transcriptase (Life Technologies, Carlsbad, CA) were added, and samples were incubated at 40°C for 2 h. The labeled cDNA was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA) as recommended by the manufacturer except that five subsequent washes were performed using the PB wash buffer. Labeling efficiencies were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

gDNA labeling

D. alkenivorans AK-01 gDNA was labeled using a BioPrime DNA Labeling System (Life Technologies, Carlsbad, CA) with the following modifications: the nucleotide mix consisted of 93 µl of RNAse free water, 2 µl of dATP, 2 µl of dGTP, 2 µl of dCTP and 1 µl of dTTP (100 mM stocks, Life Technologies, Carlsbad, CA). Labeling reactions were established in 50-µl reaction volumes using 1 µg of gDNA (100 ng μl^{-1}), 20 µl of random primers (750

μg ml^{-1} random octamers), 2 μl (40 U μl^{-1}) of Klenow fragment, 5 μl of dNTP mix and 1 μl of 1mM Cy3-dUTP in 1mM phosphate buffer (GE Healthcare, Piscataway, NJ). Reactions were incubated at 37°C for 3 h, and then terminated by incubation at 90°C for 10 min. Labeled DNA was purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA).

Microarray hybridization and scanning

For each array, Cy3-labeled gDNA (100 ng) and Cy5-labeled aRNA (2.2 μg) were combined and suspended in 10 μl of hybridization solution containing 40% formamide, 3X saline-sodium citrate (SSC: 1X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 10 μg of unlabeled herring sperm DNA, 0.1% SDS and 2.68 μl of a different tracking control. Samples were denatured at 95°C, cooled to 65°C and directly deposited onto the NimbleGen arrays. Hybridizations were conducted in a MAUI Hybridization Station (BioMicro systems Inc., Salt Lake City, UT) at 42°C for 18 h. After hybridization, microarrays were washed using NimbleGen Wash Buffer reagents (Roche NimbleGen, Madison, WI), dried (NimbleGen Microarray Dryer, Roche Applied Science, Indianapolis, IN) and scanned using a ScanArray Express microarray scanner (Perkin Elmer, Boston, MA).

Microarray analysis

Scanned images were processed and analyzed using the Nimblescan software (Roche, Madison, WI). After quantifying the spot signal intensity and background signals, signal-to-noise ratios (SNR) were calculated for each spot using the following formula: SNR = (spot signal-background signal)/(standard deviation of background signal). Spots with SNR < 2 or without enough replicates were eliminated, whereas spots with SNR > 2 were considered positive and were included in the analysis. The cDNA signals for a particular probe were normalized among the three replicates based on the gDNA signals for those three replicates. The differences in gene expression between *n*-hexadecane and hexadecanoic acid treatment conditions were determined by calculating the normalized log ratio according to a previously published formula (Mukhopadhyay *et al*. 2006):

$$\log_2 \mathrm{R} = \log_2 (\mathrm{treatment}) - \log_2 (\mathrm{control}) \quad (1)$$

The significance of the normalized log ratios was evaluated based on the Z-score calculated as previously described (Mukhopadhyay *et al*. 2006):

$$Z = \frac{\log_2 R}{\sqrt{0.25 + \sum \mathrm{variance}}} \quad (2)$$

Here, the value 0.25 is a pseudovariance term, and the variance is the sum of the standard deviations of signals from each treatment or control sample. The log ratios were further standardized using the following equation, and the Z-score was calculated using the standardized log ratios:

$$\log_2 R' = \frac{\log_2 R - \text{average}(\log_2 R)}{\sqrt{\text{variance}\,(\log_2 R)}} \quad (3)$$

Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2844.

RESULTS

Cotranscription experiments

To obtain information with respect to the potential cotranscription of other genes in *ass* gene cluster 1, RNA was extracted from AK-01 cultures grown on either *n*-hexadecane or *n*-heptadecane, and RT-PCR of the intergenic regions of 14 genes surrounding *assA1* (Table S1, Supporting Information) was conducted. These experiments yielded evidence for transcript across 11 intergenic regions that make up the putative *ass* gene cluster 1 (JGI gene locus tags Dalk_1724 through Dalk_1735), but did not indicate transcript across the intergenic regions immediately upstream and downstream of Dalk_1724 and Dalk_1735, respectively (Fig. 1b). The 12 cotranscribed genes encode the small and large subunits of methylmalonyl-CoA mutase, a putative LAO/AO ATPase, a putative MmgE/PrpD protein, the ASS activase (AssD), the putative subunits of ASS (AssB, AssC, AssA and a protein similar to MasE), a hypothetical protein, a putative chaperone protein (AssE1) and an uncharacterized protein (AssF1).

Growth conditions that would yield significant upregulation of genes in the *ass* gene cluster 2 were not identified in this study, even though the complete known alkane substrate range of AK-01 was tested (see below). Complementary cotranscription experiments for the *ass* gene cluster 2, therefore, were not pursued at this time. Further investigation of *D. alkenivorans* AK-01 under alternative substrate and/or growth conditions is therefore needed to define the roles of genes in *ass* gene cluster 2 in hydrocarbon metabolism and to define the operon structure.

RT-qPCR of *ass* gene clusters 1 and 2 in *D. alkenivorans* AK-01 incubated with *n*-hexadecane versus hexadecanoic acid

Transcription of individual genes in both *ass* gene clusters in AK-01 (Fig. 1b) was investigated in cells grown on hexadecane versus hexadecanoic acid via RT-qPCR. With respect to *ass* gene cluster 1 (as defined by cotranscription experiments), the relative expression levels of all but one gene (Dalk_1725 – Methylmalonyl-CoA mutase, large subunit) were significantly greater in

hexadecane-amended cultures (Fig. 2a and Table 1a). Among the important observations was the induction of genes that encode the putative ASS enzyme. Consistent with Callaghan *et al*. (2008), the gene encoding the catalytic subunit of ASS, *assA1*, was significantly upregulated, with transcript levels 44-fold greater in *n*-hexadecane-grown cells compared to those incubated with hexadecanoic acid. Transcript levels of *assB1*, *assC1* and Dalk 1732, which encodes a protein similar to MasE in '*Aromatoleum*' strain HxN1 (Grundmann *et al*. 2008), were 13-, 26- and 41.8-fold higher in *n*-alkane-grown cells versus fatty-acid-grown cells, respectively (Fig. 2a, Table 1a). In contrast, the gene encoding the ASS activase (*assD1*) demonstrated transcript levels that were only 2.5-fold greater (*P*-value = 0.02) in *n*-hexadecane-grown cultures as compared to fatty acid controls, which was the lowest fold change among all genes in *ass* gene cluster 1.

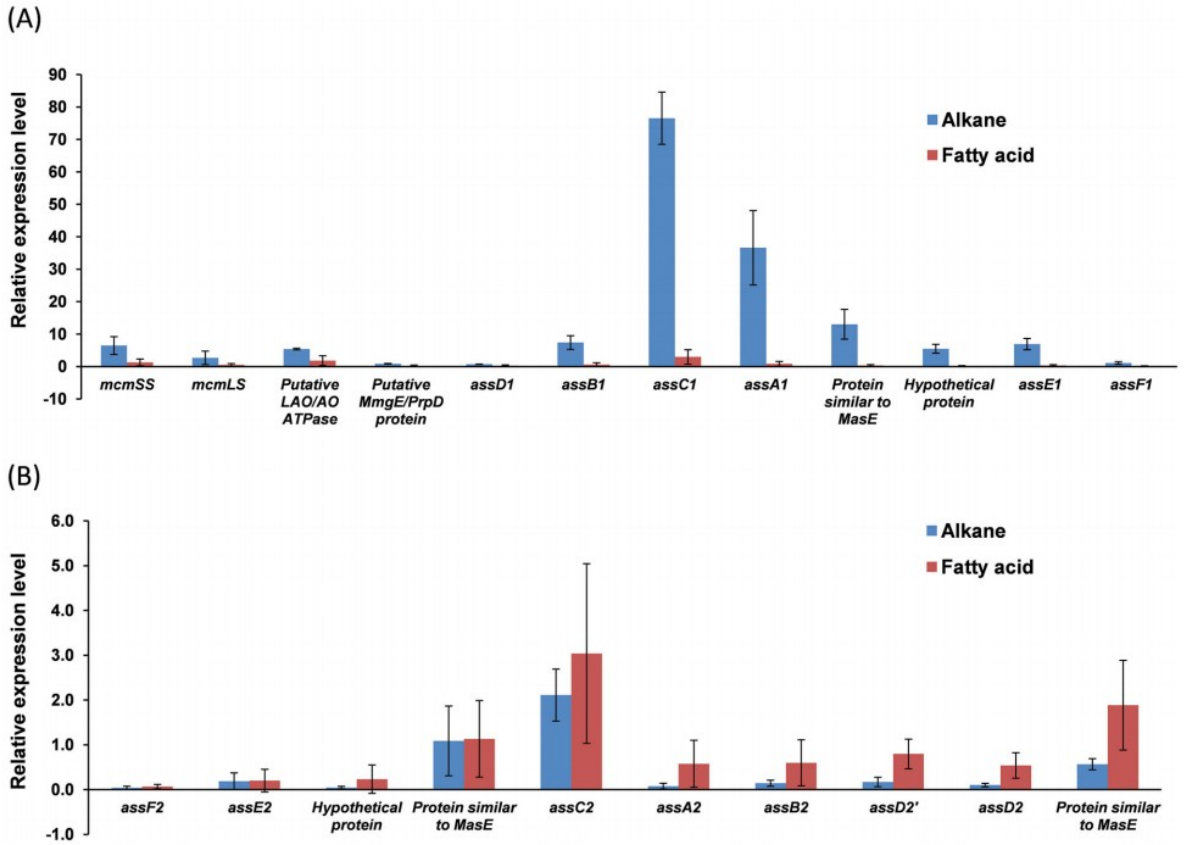

Figure 2. RT-qPCR expression of genes in *ass* gene cluster 1 (**A**) and *ass* gene cluster 2 (**B**) in *D. alkenivorans* AK-01 cells incubated with either *n*-hexadecane or hexadecanoic acid. Expression levels are shown as fold increases, relative to expression levels of *gyrB*. Error bars represent the standard deviations of three biological replicates. Fold increases of expression between the two treatments and *P*-values are reported in Table 1. Significant *P*-values are shaded in gray.

Compared to the genes in *ass* gene cluster 1, the genes in cluster 2 demonstrated lower relative expression levels (Fig. 2b, Table 1b). Furthermore, expression levels of genes in cluster 2 were higher in hexadecanoic-acid-grown cultures relative to hexadecane-grown cultures suggesting that there may be some level of downregulation in the *n*-alkane treatments. Based on the transcription levels of genes in both clusters, it

appears that *ass* gene cluster 1 is clearly induced under *n*-hexadecane-amended conditions, whereas *ass* gene cluster 2 is not.

Table 1. RT-qPCR fold increases of expression and P-values of genes in *ass* gene cluster 1 (A) and *ass* gene cluster 2 (B) in *D. alkenivorans* AK-01 cells incubated with either *n*-hexadecane or hexadecanoic acid. Significant P-values are shaded in gray.

JGI gene locus tag	Protein annotation/description	Fold increase in expression	P-value (t-test)
	(A) Alkylsuccinate synthase cluster 1		
Dalk_1724	Methylmalonyl-CoA mutase, small subunit (McmSS)	5.04	0.03
Dalk_1725	Methylmalonyl-CoA mutase, large subunit (McmLS)	5.29	0.15
Dalk_1726	Putative LAO/AO transport system ATPase	2.96	0.01
Dalk_1727	Putative MmgE/PrpD family protein	3.71	0.009
Dalk_1728	Alkylsuccinate synthase (I) glycyl radical activating enzyme (AssD1)	2.5	0.02
Dalk_1729	Alkylsuccinate synthase (I), putative beta subunit (AssB1)	12.9	0.005
Dalk_1730	Alkylsuccinate synthase (I), putative gamma subunit (AssC1)	25.9	0.0001
Dalk_1731	Alkylsuccinate synthase (I), alpha subunit (AssA1)	44	0.005
Dalk_1732	Putative uncharacterized protein similar to MasE	41.8	0.008
Dalk_1733	Hypothetical protein	30.1	0.002
Dalk_1734	Chaperone (AssE1)	20.6	0.002
Dalk_1735	Putative uncharacterized protein (AssF1)	10	0.01
	(B) Alkylsuccinate synthase cluster 2		
Dalk_2194	Putative uncharacterized protein (AssF2)	0.6	0.48
Dalk_2195	Chaperone (AssE2)	0.9	0.94
Dalk_2196	Hypothetical protein	0.2	0.35
Dalk_2197	Putative uncharacterized protein similar to MasE	0.9	0.94
Dalk_2198	Alkylsuccinate synthase (II), putative gamma subunit (AssC2)	0.7	0.48
Dalk_2199	Alkylsuccinate synthase (II), alpha subunit (AssA2)	0.1	0.17
Dalk_2200	Alkylsuccinate synthase (II), putative beta subunit (AssB2)	0.2	0.2
Dalk_2201	Alkylsuccinate synthase (II) glycyl radical activating enzyme (AssD2′)	0.2	0.03*
Dalk_2202	Alkylsuccinate synthase (II) glycyl radical activating enzyme (AssD2)	0.2	0.06
Dalk_2203	Putative uncharacterized protein similar to MasE	0.3	0.08

*Note: The level of significance is due to higher transcript levels in *hexadecanoic-acid-grown* cells.

RT-qPCR of *ass* gene clusters 1 and 2 in *D. alkenivorans* incubated with *n*-heptadecane versus heptadecanoic acid

The above experiment was repeated using cDNA from cultures grown with a C-odd alkane (*n*-heptadecane) versus the corresponding C-odd fatty acid (heptadecanoic acid) to compare patterns of transcriptional activity to those observed for C-even substrates. In general, the expression levels under heptadecane-amended conditions (Fig. S1, Supporting Information) exhibited similar patterns as observed under hexadecane-amended conditions (Fig. 2). However, there were fewer genes in cluster 1 (*assB1*, *assC1* and *assA1)* that significantly responded to the presence of heptadecane as a substrate compared to growth on hexadecane. This finding was attributed to the large standard deviations that resulted from the inherent variability in growth of these cultures, even when additional replicates were investigated (data not shown). However, the relatively high expression levels of genes under alkane-amended conditions compared to fatty-acid-amended conditions, in conjunction with the large increases in expression between treatments, suggest that *ass* gene cluster 1 is also induced when AK-01 is growing on *n*-heptadecane. In contrast, the genes in cluster 2 had very low transcript levels in the *n*-heptadecane and heptadecanoic acid treatments, and none of the transcript levels differed significantly between the two treatments (Fig.

S2, Supporting Information), suggesting that *ass* gene cluster 2 is not induced during growth on *n*-heptadecane.

RT-qPCR of *assA1* and *assA2* for other alkanes and fatty acids

Because of the proposed importance of the catalytic subunit of ASS in *n*-alkane activation (Callaghan *et al*. 2008; Grundmann *et al*. 2008), *assA1* and *assA2* genes were targeted via RT-qPCR experiments for the remaining alkanes within AK-01's substrate range (C_{13}, C_{14}, C_{15} and C_{18}) and their corresponding fatty acids. For *n*-tridecane, *n*-pentadecane and *n*-octadecane, the relative expression levels of *assA1* were significantly higher than the cultures incubated with the corresponding fatty acid substrates, while differences in expression levels for *n*-tetradecane were only marginally significant (*P*-value = 0.09) (Fig. S3, Supporting Information). Variability in growth patterns was repeatedly observed among the tetradecane-grown cultures, even when additional biological replicates were included (data not shown). When *assA2* was investigated, no upregulation was observed for *n*-tridecane, *n*-tetradecane, *n*-pentadecane or *n*-octadecane relative to their corresponding fatty acid control substrates (Fig. S3, Supporting Information).

Determination of putative transcriptional start sites

Given the slow growth of AK-01 and low RNA yields, 5′-RACE was used, *in lieu* of other methods, to investigate putative transcriptional start sites associated with *ass* gene cluster 1. Based on the cotranscription experiment (above), it was predicted that a putative transcriptional start site might be located upstream of Dalk_1724 (*mcmSS*), which encodes the small subunit of methylmalonyl-CoA mutase. Similarly, given RT-qPCR data and the importance of the putative ASS subunits, it was also hypothesized that transcriptional start sites might be located upstream of *assC1, assB1* and/or *assA1.* 5′-RACE experiments on two biological replicates of *n*-hexadecane-grown cultures revealed two transcriptional start sites. Both detected start sites are adenine bases. One start site is located 33 bp upstream of the *mcmSS* start codon, and the other is located 103 bp upstream of the *assC1* start codon, within the coding region of *assB1* (Fig. 3a). Transcriptional start sites were not identified upstream of *assA1* or *assB1*.

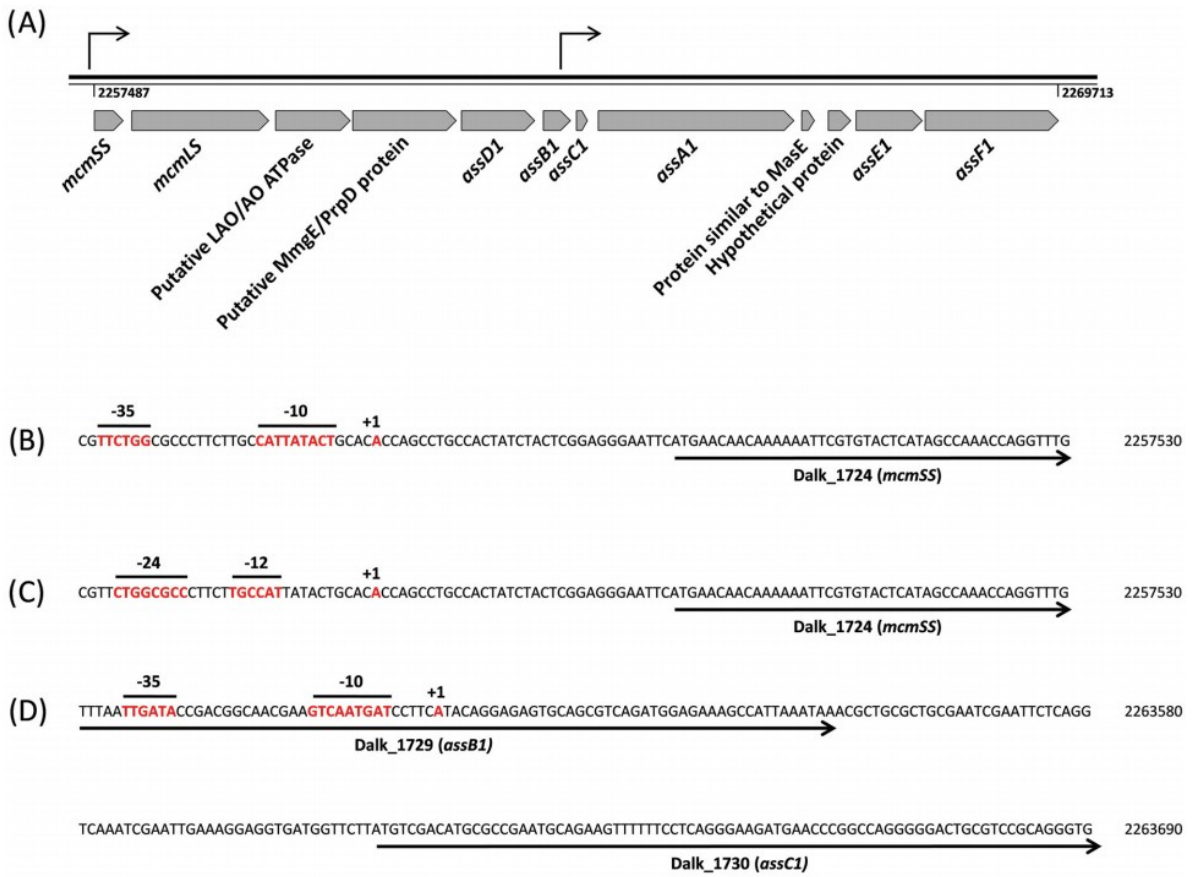

Figure 3. (A) Approximate locations of the putative transcriptional start sites (bent arrows) determined via 5′-RACE. (B) Transcriptional start sites (designated as +1) and locations of potential promoter elements analogous to *E.coli* σ^{70} binding sites (−10, −35) upstream of *mcmSS*, (C) promoter elements analogous to σ^{54} binding sites (−12, −24) upstream of *mcmSS* (D) and promoter elements analogous to *E. coli* σ^{70} binding sites (−10, −35) upstream of *assC1*.

Regions immediately upstream of the putative transcriptional start sites were searched for possible σ^{70} and σ^{54} promoter sequences using BPROM (Softberry, Inc., Mount Kisco, NY) (Solovyev and Salamov 2011) and iPro54-PseKNC software (Lin *et al*. 2014), respectively. Regions that resemble the conventional −10 (TATAAT) and −35 (TTGACA) sequences of the *E. coli* σ^{70} binding site were not detected immediately upstream of *mcmSS* (Dalk_1724) using BPROM. However, manual inspection identified regions that potentially resemble these sequences (Fig. 3b). A search for potential σ^{54} promoter sequences located at approximately −12 bp (TGC[AT][TA]) and −24 bp ([CT]TGGCA[CT][GA]) using iPro54-PseKNC software revealed a putative region at −16 bases, and manual inspection identified a putative binding site at −29 bp (Fig. 3c). With regard to *assC1* (Dalk_1730), the BPROM software predicted σ^{70} binding sites at −35 and −10 bp (Fig. 3d).

Microarray analysis

To obtain a whole-genome transcriptomic view of alkane metabolism in *D. alkenivorans* AK-01 and to provide a list of candidate genes that may be involved in the further degradation of the methylalkylsuccinic acids, a microarray experiment was performed using *n*-hexadecane-grown cells

versus hexadecanoic-acid-grown cells. A total of 4714 genes did not show differential expression (i.e. $2 > \log_2 R' > -2$) between the two treatment conditions. A total of 79 transcripts were significantly upregulated in *n*-hexadecane-grown cells compared to hexadecanoic-acid-grown cells with a fold increase (standardized $\log_2 R'$) of 2 or higher and a standardized Z-score (absolute value) of 2 or higher. Additionally, the threshold of standardized $\log_2 R'$ was lowered to 1.5 to investigate the genes that demonstrated 'marginal' upregulation (i.e. $1.5 < \log_2 R' < 2$) (Table S4, Supporting Information); a total of 131 genes were found to be marginally upregulated. The number of downregulated genes (i.e. $\log_2 R' \leq -2$) in *n*-hexadecane-amended conditions compared to hexadecanoic-acid-amended conditions was 136 (Table S5, Supporting Information).

Upregulated gene expression in *D. alkenivorans* AK-01 grown on n-hexadecane

Consistent with RT-qPCR data, genes encoding the putative subunits of ASS in *ass* gene cluster 1 (*assA1*, *assB1, assC1* and Dalk_1732) were significantly upregulated (Fig. 4; Table S6, Supporting Information). Surprisingly, *assD2′* and *assC2* were also identified among the upregulated genes (Table S7, Supporting Information), but these observations are not supported by RT-qPCR (Table 1b; Table S8, Supporting Information). Given that RT-qPCR is the more sensitive method, these results should be interpreted with caution. Although not indicated by *in silico* analysis (data not shown), it is possible that cross-hybridization of *assD1* and *assC1* may have impacted microarray signals of their homologs in *ass* gene cluster 2.

Genes potentially involved in the further degradation of methylhexadecylsuccinic acid (CoA activation, carbon-skeleton rearrangement, decarboxylation and beta-oxidation) were also upregulated. Specifically, three AMP-dependent Co-ligases were marginally upregulated, one of which (Dalk_1737) was further confirmed by RT-qPCR due to its proximity to *ass* gene cluster 1. One acyl-CoA dehydrogenase and one enoyl-CoA hydratase were upregulated based on microarray, and another enoyl-CoA dehydratase (Dalk_1736) in close proximity to cluster 1 was upregulated based on RT-qPCR. A second acyl-CoA dehydrogenase, two beta ketothiolases and one electron transfer flavoprotein were marginally upregulated. Consistent with RT-qPCR data, the expression of a gene coding for the small subunit of methylmalonyl-CoA mutase (Dalk_1724) was significantly upregulated (Fig. 4; Table S4, Supporting Information), whereas the expression of the gene coding for the large subunit of methylmalonyl-CoA mutase (Dalk_1725) was found to be marginally upregulated. Microarray data also demonstrated upregulation of a putative methylmalonyl-CoA mutase metallochaperone (Dalk_0222). Interestingly, marginal upregulation was observed for Dalk_1740 (Table S4, Supporting Information), which encodes a putative decarboxylase and is located downstream of *ass* gene cluster 1. Verification by RT-qPCR experiments showed significant upregulation, with transcript levels 17-fold greater ($P = 0.002$) in *n*-

hexadecane-grown cells compared to those incubated with hexadecanoic acid (Table S8, Supporting Information). Finally, in addition to genes specifically related to anaerobic alkane metabolism, several other genes were significantly induced ($\log_2 R' \geq 2$) (Table S4, Supporting Information) and included 20 hypothetical proteins, a putative pyruvate formate lyase, an iron-containing alcohol dehydrogenase, two NADH:flavin oxidoreductase/NADH oxidases and one radical SAM domain protein.

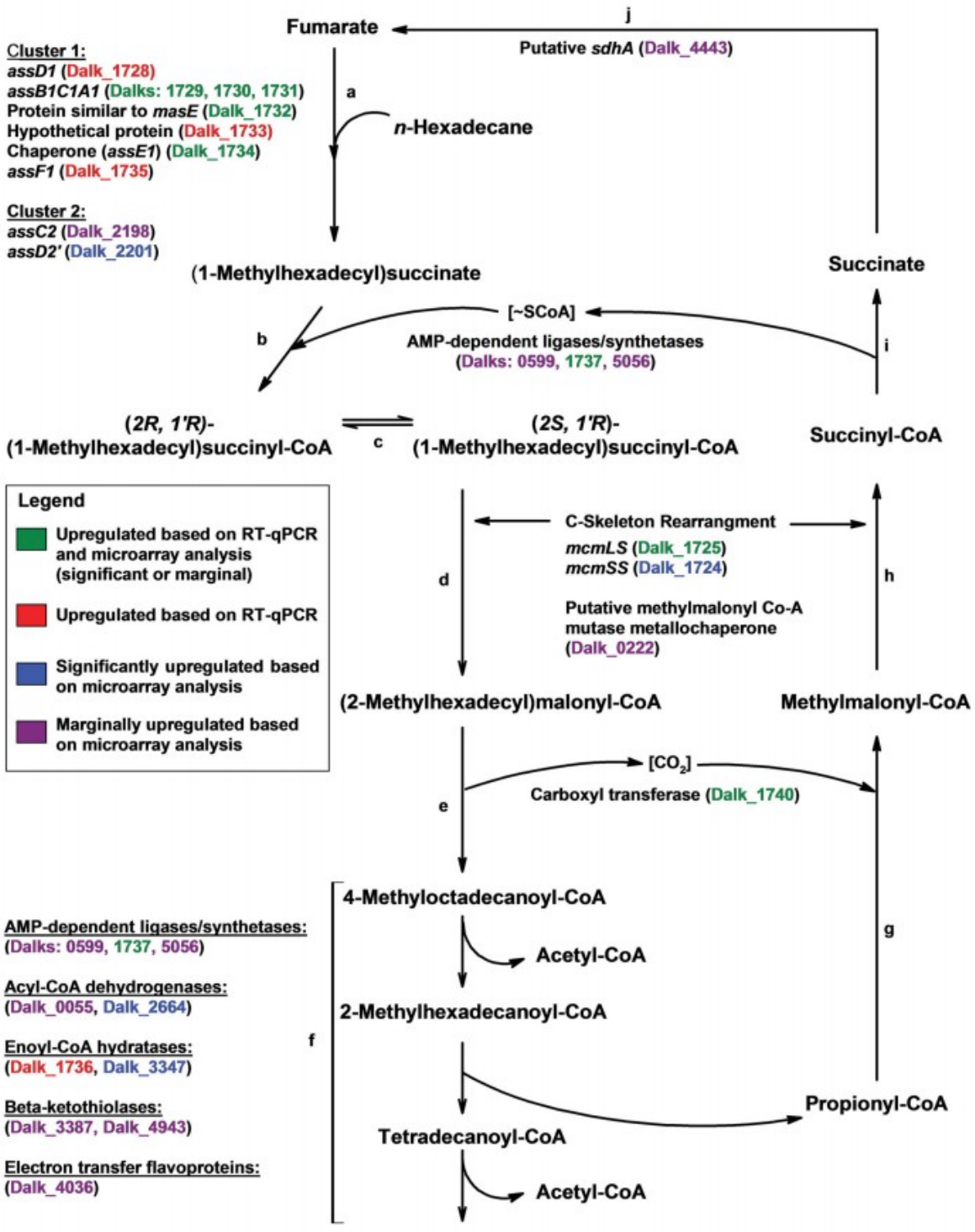

Figure 4. Proposed mechanism of anaerobic *n*-hexadecane degradation in *D. alkenivorans* AK-01 based on metabolite profiling (Callaghan *et al.* 2006) and upregulation of genes hypothesized to be involved in alkane activation and degradation based on RT-qPCR analysis and microarray analysis (this study). Hypothesized proteins catalyzing the requisite reactions: a—alkylsuccinate synthase; b—(1-methylalkyl)succinate-CoA ligase; c—(1-methylalkyl)succinyl-CoA epimerase; d—(2-methylalkyl)malonyl-CoA mutase; e—(2-methylalkyl)malonyl-CoA decarboxylase; f—beta-oxidation proteins (e.g. fatty acyl-CoA dehydrogenases, 3-hydroxyacyl-CoA hydrolyases (syn. enoyl-CoA hydratases), 3-hydroxyacyl-CoA dehydrogenases and ß-ketothiolases (syn. acetyl-CoA acetyltransferases)); g—propionyl-CoA carboxylase; h—methylmalonyl-CoA mutase; i—succinate thiokinase; and j—succinate dehydrogenase. Gene abbreviations: *ass*—alkylsuccinate synthase; *mcmLS*—methylmalonyl-CoA mutase large subunit; *mcmSS*—methylmalonyl-CoA mutase small subunit; and *sdhA*—succinate dehydrogenase alpha subunit.

Downregulation of genes in *D. alkenivorans* AK-01 grown on n-hexadecane

Thirty-eight hypothetical proteins were significantly downregulated in cells incubated with *n*-hexadecane (Table S5, Supporting Information). Six genes associated with beta-oxidation were marginally downregulated including four acyl-CoA dehydrogenase domain proteins, an electron transfer flavoprotein beta subunit and an AMP-dependent CoA ligase/synthase. Interestingly, there were several genes downregulated that encode proteins for assimilatory and dissimilatory sulfate reduction (i.e. sulfate adenylyltransferase, an Na(+)-dependent di- and tricarboxylate transporter/sulfate sodium cotransporter, the alpha and beta subunits of adenylylsulfate reductase, the dissimilatory sulfite reductase complex-associated protein DsrD, and the alpha and beta subunits of dissimilatory sulfite reductase), as well as 4 genes encoding subunits of ATP synthase and 10 genes encoding ribosomal structural and associated proteins, transcriptional regulators, translation machinery and signal transduction. Finally, 12 genes that may play a role in oxygen/radical detoxification including rubredoxins, peroxiredoxins, rubrerythrins, alkyl hydroperoxide reductases, flavodiiron proteins and cytochromes were downregulated.

DISCUSSION

Several studies on *aerobic* alkane degradation have demonstrated differential expression of multiple copies of monooxygenases/alkane hydroxylases due to different growth stages or alkane substrate range (Hamamura, Yeager and Arp 2001; Tani *et al*. 2001; Marín, Yuste and Rojo 2003). Despite our hypothesis that differential gene expression would be observed in AK-01 depending on the alkane substrate utilized, the RT-qPCR and microarray data only showed clear induction of *ass* gene cluster 1 across AK-01's known alkane substrate range. Given AK-01's metabolic versatility (So and Young 1999a), *ass* gene cluster 2 might be involved in the utilization of alternative hydrocarbon substrates and/or is expressed under physiological conditions that were not tested herein. Preliminary data suggest that *ass* gene cluster 2 is also not involved in 1-alkene degradation in AK-01 (Callaghan, unpublished data). Given recent work demonstrating the activation of *iso*-alkanes via ‘fumarate addition’ under methanogenic conditions (Abu Laban *et al*. 2015), it may be important to test AK-01 for growth on *iso*-alkanes as well. Alternatively, *ass* gene cluster 2 may play a role under syntrophic conditions, given that AK-01 is capable of alkane utilization in the absence of sulfate in co-culture with *Methanospirillum hungatei* JF-1 (Callaghan *et al*. 2012).

Interestingly, detectable transcription levels of *ass* genes were also measured in some fatty-acid-grown cells, suggesting perhaps that there is a low level of constitutive expression. Such a finding may be analogous to the *mas* operon in ‘*Aromatoleum*’ sp. HxN1, which is induced by a wide range of hydrocarbons and alcohols (Webner 2012). It has been postulated that this is due to relaxed specificity of the sensor, which could enable

immediate use of *n*-alkanes for energy gain once they have entered the cell (Webner 2012). As discussed in Webner (2012), relaxed substrate specificity has also been reported for other regulators/sensors involved in hydrocarbon degradation (de Lorenzo and Perez-Martin 1996; Shingler 2003). Alternatively, low-level expression of *ass* genes during growth on fatty acids may be due to the requirement of fatty acids or other downstream metabolites for full induction of the *ass* 1 operon, similar to the requirement of benzylsuccinate for full induction of the *tutE tutFDGH* gene complex in *Thauera aromatica* T1 (Coschigano and Bishop 2004).

Cotranscription, 5′-RACE and RT-qPCR data

Although cotranscription experiments described here cannot exclude the possibility that requisite genes in *ass* gene cluster 1 are cotranscribed via more than a single transcript, they allowed constraining of the putative *ass* 1 operon and provided a baseline for determining which genes should be investigated via RT-qPCR. The results from 5′-RACE experiments support the hypothesis that there may be at least two transcripts generated for *ass* gene cluster 1. The presence of more than one putative transcript is consistent with observations of the *mas* operon identified in '*Aromatoleum*' HxN1 (Grundmann *et al*. 2008), in which two *mas* gene transcripts were detected. Similar observations have been made for several characterized *bss* operons in which more than one transcript have either been detected or proposed (Coschigano 2000; Achong, Rodriguez and Spormann 2001; Hermuth, Leuthner and Heider 2002; Kube *et al*. 2004). Additionally, the possibility that the second putative transcript in AK-01 is generated by endonucleolytic processing cannot be excluded. The latter was observed for the *bss* operon in *T. aromatica* strain K172 (Hermuth, Leuthner and Heider 2002).

Together, the cotranscription and RT-qPCR data are consistent with the hypothesis that Dalks 1729–1732 encode a four-subunit ASS (Callaghan *et al*. 2008; Grundmann *et al*. 2008; Webner 2012) and are supported by recent investigations of MAS in '*Aromatoleum*' sp. HxN1 (Webner 2012). Although the specific roles of the requisite subunits have not been clearly defined, studies of BSS have provided some insight. Given the conserved glycine and cysteine motifs identified in all GREs to date (for review, see Selmer, Pierik and Heider 2005), AssA1 is proposed to be the catalytic subunit of ASS required for alkane activation (Callaghan *et al*. 2008; Grundmann *et al*. 2008). The annotation of the remaining ASS subunits is less clear and is still being debated. AssC1 shares ~34 to ~38% sequence identity with BssC from well-characterized organisms (e.g. *Azoarcus* sp. T., *T. aromatica* K172 and '*Aromatoleum*' *aromaticum* EbN1, etc.) and contains similar FeS cluster-binding motifs identified in BssC. A previous study of the subunit structure of BSS revealed that FeS clusters are associated with the beta and gamma subunits and that coexpression of the gamma subunit (but not the beta subunit) is essential for efficient expression of the alpha subunit, suggesting that the gamma subunit plays a role in folding and stabilizing the catalytic subunit of BSS (Li *et al*. 2009). Subsequent work by others also identified FeS

clusters that are likely located in the beta and gamma subunits of BSS, and the authors of that study further proposed that these clusters may be involved in electron transfer during the generation of the glycyl-radical site and in coordinating binding of the carboxyl groups of fumarate (Hilberg *et al*. 2012). Therefore, it is possible that the product of *assC1* (i.e. gamma subunit of ASS) in AK-01 performs the same or a similar function in the assembly and functioning of ASS. With respect to BssB, it has been suggested that this protein could act as a regulator of activation and may additionally be involved in regulating access to the active site of BSS (Funk *et al*. 2014). For known ASS/MAS proteins, however, it still remains unclear whether the genes annotated as *assB/masB* (e.g. *assB1* (Dalk_1729) and *assB2* (Dalk_2200) in AK-01; *masB* in '*Aromatoleum*' strain HxN1) are analogous to *bssB*. The initial annotation of these requisite proteins in AK-01 relied on the similarities to BssB based on FeS cluster-binding motifs (Callaghan *et al*. 2008). However, subsequent annotation of the *ass* gene clusters revealed that there were also hypothetical proteins (Dalk_1732, Dalk_2197 and Dalk_2203) (Callaghan *et al*. 2012) that share similarity (ranging from ~29 to 64% amino acid identity) to MasE in HxN1 (Grundmann *et al*. 2008), which was originally proposed to be analogous to BssB based on size and FeS cluster-binding motifs (Grundmann *et al*. 2008). More recent investigations of MAS in HxN1 have revealed that MasB, MasC, MasD and MasE are protein subunits (Webner 2012), but it is still undetermined whether AssB/MasB or the MasE/MasE-like proteins are the analogous beta subunits. Regardless, transcript levels of both *assB1* and the gene encoding a hypothetical protein similar to MasE (Dalk_1732) were significant in the study herein, suggesting that they are important components of alkylsuccinate synthase.

All GREs require activation via an activase that abstracts a hydrogen atom from the alpha carbon of a highly conserved glycine residue to generate a glycyl radical. The glycyl radical then abstracts a hydrogen atom from a conserved cysteine residue, forming the thiyl radical that is involved in substrate activation (Shisler and Broderick 2014). Interestingly, transcript levels of *assD1* were a lot lower than those for the ASS subunit genes. This finding is potentially explained by the fact that GRE-activating enzymes do not need to be produced in a 1:1 stoichiometric abundance with their respective target GREs. For example, in a study of *T. aromatica* strain K172 grown on toluene, the molar ratio of monomers of BSS and the activating enzyme (BssD) was estimated to be 14:1 (Hermuth, Leuthner and Heider 2002). Similarly, in *E. coli*, the transcription of structural genes of pyruvate formate-lyase, another well-studied GRE, occurs separately from transcription of its activating protein and at a different rate (Sauter and Sawers 1990).

Microarray analysis

To date, the focus of anaerobic alkane metabolism has mainly been on the activation step. The study herein also aimed to examine processes related to

alkane uptake and the further degradation of methylalkylsuccinic acids. Following uptake, the proposed pathway of *n*-alkane oxidation in *D. alkenivorans* AK-01 proceeds via subterminal addition of the alkane to the double bond of fumarate (Callaghan *et al*. 2006) (Fig. 4), presumably catalyzed by alkylsuccinate synthase (Callaghan *et al*. 2008). It is assumed that the resulting methylalkylsuccinate is then converted to its CoA thioester and that the isomer of 1-methylalkylsuccinyl-CoA initially formed by addition of the alkane to fumarate is epimerized to generate the (*2S*, *1′R*) diastereoisomer (Jarling *et al*. 2012). Subsequent degradation proceeds via carbon-skeleton rearrangement, decarboxylation and beta-oxidation, wherein fumarate can be regenerated from propionyl-CoA (Wilkes *et al*. 2002; Callaghan *et al*. 2006). Whole-genome microarray data were analyzed in the context of this proposed pathway and compared to the RT-qPCR data (Fig. 4 and Table S8, Supporting Information).

Bacterial uptake of alkanes is thought to be enhanced by several mechanisms including passive diffusion, secretion of biosurfactants or conformational changes in outer membrane proteins (for reviews, see Rojo 2010; Wang and Shao 2013). Based on AK-01's genome inventory, AK-01 does not contain genes with significant homology to those involved in biosurfactant production such as esterase (*est*), rhamnosyltransferase (*rhl*), lipase (*lip*), lichenysin synthetase (*lch*) or surfactin synthetase (*sfp*) (Sullivan 1998). Very weak homology to a putative lipase (Dalk_3606) and a lipase chaperone protein (Dalk_4747) suggests that biosurfactant production is likely not an important mechanism that facilitates alkane uptake in AK-01. With respect to transporters, the only transporter-related protein that was upregulated based on microarray data was a TonB family protein (Dalk_3600). Thus far, TonB-dependent transporters have not been implicated in the uptake of hydrocarbons (Schauer, Rodionov and de Reuse 2008). Further analysis of the AK-01 genome did not reveal a homolog to the alkane-induced outer membrane lipoprotein (Blc) (NCBI accession no. CAL16317) identified in *Alcanivorax borkumensis* SK2 (Sabirova *et al*. 2011). It has been hypothesized that this protein could mediate alkane transport because it contains a lipocalin-like domain, which can facilitate the formation of hydrophobic pockets that allow the transport of small hydrophobic molecules (Pervaiz and Brew 1987). Interestingly, a survey of AK-01's genome for proteins similar to FadL in *E. coli* K-12(P10384) and FadL homologs, such as those found in *A. dieselolei* B5 (AFT71821) (Lai, Li and Shao 2012), did reveal what may be a FadL-like protein (Dalk_0288). Dalk_0288 is annotated as a membrane protein involved in aromatic hydrocarbon degradation (Transport classification: TC:1.B.9 - The FadL Outer Membrane Protein (FadL) Family; KO:K06076 *fadL* long-chain fatty acid transport protein). FadL proteins are found in various xenobiotic-degrading microorganisms and are involved in the transport of hydrophobic compounds (van den Berg *et al*. 2004, 2005; Hearn *et al*. 2009). Microarray analysis does not indicate differential expression of this gene. However, it should be noted

that this protein could potentially be used for the uptake of both hexadecanoic acid and *n*-hexadecane, and therefore, may not be subject to a regulatory response induced by the presence of an alkane.

Based on both RT-qPCR and microarray data, the activation of *n*-hexadecane is likely catalyzed by ASS encoded by *ass* operon 1 (Fig. 4; Table S8, Supporting Information), and may require a chaperone protein (*assE1*) homologous to TutH in *T. aromatica* strain T1, BssE in *Azoarcus* sp. strain T and BssE in *T. aromatica* strain K172 (Coschigano 2000; Achong, Rodriguez and Spormann 2001; Hermuth, Leuthner and Heider 2002). It has been proposed that this protein may function as an ATP-dependent chaperone for assembly and/or activation of BSS (Hermuth, Leuthner and Heider 2002) and perhaps plays an analogous role with regard to ASS. In line with the assumption that methylalkylsuccinate is converted to its CoA thioester, three AMP-dependent Co-ligases were marginally upregulated based on microarray analyses, one of which (Dalk_1737) was confirmed by RT-qPCR. Alternatively, it has been proposed that CoA activation and subsequent epimerization of the methylalkylsuccinic acid diastereoisomer could be catalyzed by the same enzyme, such as α-methylacyl-CoA racemase or a homolog belonging to the family III CoA-transferases (Heider 2001; Jarling *et al*. 2012). A genome survey for genes similar to family III CoA transferases (i.e. (*R*)-benzylsuccinate CoA transferase (BbsE, BbsF), oxalate CoA transferase (Frc), (*R*)-phenyllactate CoA transferase (PLCT), (*R*)-carnitine CoA transferase (CaiB), putative cholate CoA-transferase (BaiF) and 2-methyl-CoA racemase)) (Heider 2001) revealed one gene (Dalk_0909), which is annotated as an L-carnitine dehydratase/bile acid inducible protein F (E.C. 5.1.99.4 – alpha-methylacyl-CoA racemase). However, this gene was not found to be differentially expressed under the tested conditions.

To date, the enzyme catalyzing the carbon-skeleton rearrangement of methylalkylsuccinic acids has not been identified. It has been postulated that the requisite enzyme might be similar to methylmalonyl-CoA mutase (Wilkes *et al*. 2002), which catalyzes the reversible transformation of succinyl-CoA to (*R*)-methylmalonyl-CoA (for review, see Ludwig and Matthews 1997). Both RT-qPCR and microarray data indicated that the small and large subunits of methylmalonyl-CoA mutase were significantly and marginally upregulated, respectively (Fig. 4; Table S8, Supporting Information), and microarray data indicated marginal upregulation of a putative methylmalonyl-CoA mutase metallochaperone (Dalk_0222). These data can be interpreted in two ways. First, it is possible that the methylmalonyl-CoA mutase in AK-01 catalyzes the carbon skeleton rearrangement of larger malonates, such as methylalkylsuccinates. However, this hypothesis has not been substantiated in AK-01 or other alkane-utilizing isolates thus far. Second, the methylmalonyl-CoA pathway could be employed to convert propionyl-CoA formed during the beta-oxidation of odd-numbered fatty acids, to succinyl-CoA, which is then converted to succinate and then fumarate via succinate thiokinase (Dalk_3783 and 3784) and

succinate dehydrogenase (Dalk_4444), respectively. The latter was also found to be marginally upregulated by microarray analysis. Therefore, the upregulation of *mcmSS, mcmLS* and the *mcm* metallochaperone could possibly be attributed to one or both of these processes during anaerobic alkane degradation in AK-01.

Following carbon-skeleton rearrangement, the resulting malonate undergoes decarboxylation (Wilkes *et al*. 2002; Callaghan *et al*. 2006). Marginal upregulated gene expression was observed for Dalk_1740, which is found downstream of *ass* gene cluster 1. This finding was verified via RT-qPCR (data not shown). Dalk_1740 encodes a protein with a predicted amino acid sequence that is 47.5% identical to that of a methylmalonyl-CoA decarboxylase protein in *Archaeoglobus fulgidus*, and it has been hypothesized that this protein may play a role in the decarboxylation of the rearranged intermediate or catalyze a carboxyl transfer rather than a decarboxylation (Callaghan *et al*. 2012). With respect to the latter, carboxyl transfer from the alkylsuccinyl-CoA intermediate or another metabolite to propionyl-CoA could potentially initiate the methylmalonyl-CoA pathway for regeneration of fumarate.

Further transformation of the methylated fatty acid, 4-methyloctadecanoic acid, would proceed via beta-oxidation (Fig. 4), and several genes associated with beta-oxidation were either significantly or marginally upregulated, as determined via microarray. In contrast, there were no genes related to beta-oxidation that were significantly upregulated under fatty acid conditions; only six were marginally upregulated. These results could possibly be explained by the fact that alkane metabolism produces both branched and linear chain fatty acids, the latter being solely produced during hexadecanoic acid catabolism. The upregulation of a few specific genes related to beta-oxidation under the alkane-amended conditions could be due to a different suite of beta-oxidation genes being required for the oxidation of the branched acids.

The relative downregulation of genes related to sulfate reduction, ATP synthesis and oxygen/radical detoxification when AK-01 is grown on alkanes presents an interesting corollary potentially related to the slower growth on the more reduced substrate. Cultures grown on hexadecanoic acid typically required 2 to 3 weeks to utilize 70%–80% sulfate in the medium, whereas the cultures grown on *n*-hexadecane had to be incubated for an average of 10 to 13 weeks to achieve the same level of sulfate loss, a difference that was highly significant ($P = 0.01$). Cellular RNA content in bacteria is known to be more or less correlated with growth rate in both cultures and natural populations (Schaechter, Maaløe and Kjeldgaard 1958; DeLong, Wickham and Pace 1989; Kemp, Lee and LaRoche 1993). In addition, various mechanisms can modulate growth-rate-dependent gene expression in bacteria, including the relative rates of ribosomal protein synthesis, the transcription of transcriptional regulators or genomic arrangement relative to the origin of replication (Klumpp and Hwa 2014). Growth can also generate

feedbacks (positive or negative) for the expression of genes whose products have an effect on growth itself (Klumpp, Zhang and Hwa 2009), potentially explaining patterns of downregulation for ribosomal components, ATPase and sulfate reduction related proteins observed herein. Similarly, the downregulation of proteins involved in oxygen/radical detoxification in AK-01 may stem from a feedback that relates to the glycyl radical mechanism required for alkane activation by ASS.

CONCLUSIONS

Very little is known about alkylsuccinate synthases as they relate to hydrocarbon substrate range, and few model organisms are available to investigate these processes. Although the role of *ass* gene cluster 2 warrants further investigation, the data generated for *ass* gene cluster 1 may shed light on the transcription of *ass* genes in other model organisms. For example, the transcription of *ass* genes in *D. aliphaticivorans* CV2803^T has not been investigated, but the requisite activases and ASS subunits in CV2803^T (JGI locus tags: G491DRAFT_03717–G491DRAFT03721 and G491DRAFT_04068–G491DRAFT_04072) share between 96% and 100% amino acid identity with their homologs in AK-01. CV2803^T's substrate range includes the same complement of alkanes utilized by AK-01 (C_{13}–C_{18}) (Cravo-Laureau *et al*. 2004), suggesting that transcription patterns observed here could potentially apply to CV2803^T.

Since the discovery of alkane addition to fumarate (Kropp, Davidova and Suflita 2000), several studies have speculated about the CoA activation and subsequent epimerization of the methylalkylsuccinic acid diastereoisomer (Heider 2001; Jarling *et al*. 2012), the possible involvement of methylmalonyl-CoA mutase-like proteins in the carbon-skeleton rearrangement of methylalkylsuccinic acids and/or the regeneration of fumarate (Wilkes *et al*. 2002), and the putative protein that may catalyze the decarboxylation step (Callaghan *et al*. 2012). However, most recent investigations have focused primarily on the presence or absence of *assA*/*masD* genes in cultures and environmental samples, and the genetic basis for the key reactions that follow fumarate addition remains somewhat unresolved. In the study herein, microarray analysis of AK-01 provided evidence of genes that may be involved in the downstream carbon-skeleton rearrangement and decarboxylation reactions (i.e. methylmalonyl-CoA mutase proteins and a putative carboxyl transferase). Additional gene candidates encoding proteins that may facilitate alkane uptake (Dalk_0288, FadL-like protein) or that catalyze the CoA activation and epimerization of methylalkylsuccinic acids (Dalk_0909, putative alpha-methylacyl-CoA racemase) were also identified in the genome, but not differentially expressed under the tested conditions. Experiments that focus on alternative growth conditions may elucidate these processes. Altogether, these findings provide new and promising directions for studying the transcriptional regulation of genes involved in alkane addition to fumarate, fumarate recycling and the processing of methylalkylsuccinates in downstream

metabolism. Additionally, data presented herein have implications for molecular studies that mine ecological datasets related to hydrocarbon degradation and point to important physiological adaptations that underlie the slow-growing life-style of sulfate-reducing alkane utilizers.

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